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Essential features of the assembly origin of tobacco mosaic virus RNA as studied by directed mutagenesis

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ABSTRACT

The assembly origin of tobacco mosaic virus RNA contains three stable hairpin loops. Coat protein disks bind first to loop 1 (the 3' most) during virus assembly, but the whole region is coated in a concerted fashion even in conditions of limiting protein. It is shown by in vitro packaging assays using mutant assembly origin transcripts that rapid and specific assembly initiation occurs in the absence of loops 2 and 3, but is abolished on removal of loop 1. Deletion or alteration of the unpaired AAGAAGUCG sequence at the apex of loop 1 also abolishes rapid packaging; this sequence is therefore instrumental in disk binding. Alteration of this sequence to $(A)_9$ leads to packaging at a very low rate (half time 12 hours) which is apparently non-sequence specific. Substitution of $(CCG)_3$ evokes packaging with a half time of 3 hours, as compared to 15 seconds for the wild type assembly origin. These results suggest that the three-base G periodicity within this sequence element is an important feature in assembly nucleation.

INTRODUCTION

Tobacco mosaic virus (TMV) is a simple but highly effective plant infectious agent. It consists of a single, positive strand 6.4 kilobase (kb) RNA molecule coated in a helical virion by over 2000 copies of the viral coat protein, and has been the subject of extensive structural investigation (1,2). Since the observation that infectious TMV could be reconstituted in vitro from purified RNA and coat protein under plausible physiological conditions (3), it has also become a well-studied model virus assembly system (for reviews see (4,5)).

The initiation of TMV assembly requires the presence of a defined coat protein aggregate; the 34 subunit, two-ring disk (6)(or protohelix (7)). Assembly begins with a specific interaction between this aggregate and an internal TMV RNA sequence termed the assembly origin (O_a), located about 1 kb from the 3' end of the genome (8). The current picture of TMV assembly involves the sequential addition of disks or smaller protein aggregates to

a growing helical rod established on a loop of RNA around O_a, the free 5' end moving up the central hole of the rod to be coated as assembly proceeds (9,10).

By analysis of RNA fragments protected by coat protein disks from nuclease digestion, O_a was shown to consist of a region of about 300 bases which is coated in a concerted fashion (11,12). Shorter protected fragments of 50-100 bases could be isolated in very low yield, indicating that assembly begins at the 3' end of this region. Direct RNA sequencing revealed a tract of bases largely lacking cytosine residues which contained in places a repeat of guanosine every three bases (12,13), vindicating the prediction that the interaction of a coat protein subunit with 3 bases of RNA would be signalled by a repeating sequence of this nature (14). In particular, a stable hairpin with the sequence AAGAAGUCG exposed as a single strand at its apex, termed here loop 1, was identified as the centre of the earliest protected portion of O_a.

More recently the complete sequence of TMV RNA has become available (15), and this has been combined with an analysis of partial nuclease digestion products of O_a to produce an extended secondary structure model for the region (16), shown in Figure 1(a). A speculative picture of assembly has been proposed (16) in which the weakly base paired loops 2 and 3 are required to pass through the growing rod and reform on its 5' side to facilitate addition of further disks. This would account for disparate observations implicating an extended RNA structure in the process such as the concerted coating of O_a mentioned above, the loss of specificity of disk binding on fragmentation of TMV RNA (17,18), the similar spacing of hairpin structures in O_a to those in the pseudo-assembly origin (ψO_a, a second TMV RNA fragment which can interact with disks (19,20)) and the discovery of potentially stem weakening mutations in loop 3 (the 5' most) in an assembly defective TMV mutant (21).

This paper describes the use of an assay for the nucleation of TMV assembly using disks and RNA transcribed <u>in vitro</u> from a cDNA clone for O_a (22) to dissect the functionally important parts of the TMV assembly origin. Sequence and secondary structure elements in O_a have been altered and deleted by oligonucleotide directed mutagenesis, and the effect on RNA encapsidation by disks measured. The results shed light on the sequence and structural basis of the protein-RNA recognition process involved in the initiation of TMV assembly.

MATERIALS AND METHODS

Preparation of disks

TMV was purified from systematically infected N.tabacum by the procedure of Leberman (23). TMV coat protein was prepared from stored virus as described (24) and disks prepared by dialysis into ionic strength 0.1M sodium phosphate buffer pH 7 at 4°C, followed by incubation at 20°C overnight. Each disk preparation was tested before use by its ability either to encapsidate freshly prepared TMV RNA (25) as measured turbidimetrically (6), or to quantitatively filter bind a labelled O_a transcript (see below).

RNA preparation

Appropriately linearised pSP65 derivatives (1µg) were transcribed in 20µl of 40mM TrisHCl pH7.5, 6mM $MgCl_2$, 2mM spermidine, 10mM dithiothreitol, 0.5mM each of UTP, CTP, ATP and GTP, 20 units RNasin plus α^{32}P-UTP (800 Ci/mmol, Amersham) and 5-10 units SP6 RNA polymerase (NEN) for 1 hour at 40°C (26). ^{32}P labelled transcripts were purified in denaturing polyacrylamide gels, visualised by autoradiography and eluted overnight at 37°C in 0.6ml of 0.5M ammonium acetate, 10mM magnesium acetate, 1mM EDTA, 0.1% SDS, 10 µg/ml tRNA. Addition of carrier tRNA was found to increase recovery of transcript but not to otherwise affect the results. RNA was ethanol precipitated twice immediately before use.

Reassembly assays

Labelled O_a transcripts were reassembled with disks in 15µl of ionic strength 0.1M sodium phosphate buffer pH7; TMV protein was present at a concentration of 1-1.5 mg/ml freshly diluted from >10 mg/ml, consisting of approximately 80% disks and 20% 'A' protein (24). Under these conditions of protein excess, packaging is pseudo-first order with respect to RNA concentration (11).

For the nuclease resistance/gel assay (22), assembly was for 10 minutes at room temperature followed by the addition of 5 units micrococcal nuclease (Boehringer), $CaCl_2$ to 1mM and digestion at room temperature for 1-2 hours. Analysis was by electrophoresis in 4% polyacrylamide/8M urea gels, followed by autoradiography. For the filter binding assay (11), the standard reassembly reaction was performed for various time periods at room temperature (or at the temperature specified). A Schleicher & Schull BA85 nitrocellulose filter was washed with 1ml of phosphate buffer, the reaction diluted with 1ml of the the same buffer and filtered immediately. Filters

were washed with a further 1ml of buffer, dried and taken for liquid scintillation counting in Aquasol (NEN). Where filter binding results are quoted as '% packaging' this refers to filter bound radioactivity expressed as a percentage of the total radioactivity available for packaging in a purified transcript preparation.

Clone construction

The O_a cDNA clone consisted of TMV vulgare sequence between the Msp I sites at bases 5111 and 5546 in the vector M13mp7 (27), from a clone bank constructed by P.Goelet (15). Mutations in O_a were constructed as described (28) using the following oligonucleotides, complementary to the coding-sense M13 construct; GGACATCTTC-deletion-GGCCCTCCGT (Δ1), GTTTGTAATC-deletion-GCGGACAGAA (Δ2), AACCCGCTGA-deletion-TTTTTCATCG (Δ3) and AACTCATCAA-deletion-CTGTAAGTTC (Δ4) using O_a as the template, AACTCATCAATTTTTTTTTCTGTAAGTTC (IN1) and AACTCATCAACGGCGGCGGCTGTAAGTTC (IN2) using Δ4 as the template. All primers were synthesised on an Applied Biosystems 380B machine by T. Smith, and gel purified. The structures of deletion mutants are shown in Figure 1(b), and the sequence breakpoints marked in Figure 1(a). Constructions were confirmed by sequencing (29) and cloned into Bam HI or Eco RI linearised pSP65 (26) by standard procedures (30). Clones with the insert in the correct orientation were identified by restriction mapping and verified by subcloning into M13 vectors and re-sequencing. Plasmids could then be linearised with Hind III for transcription. The O_a RNA transcript contained all the sequence shown in Figure 1(a), plus 5' secondary structure elements (16). Together with plasmid-derived flanking sequences the transcript had a total length of 533 bases.

The coat protein sequence (including ψO_a) contained residues 5712 to 6369 of the TMV OM sequence (31), provided by M.Bevan. This fragment was cloned into pSP65 opened with Bam HI and Hind III, and the resulting construct linearised with Hinf I for transcription. A 720 bp phage λ/human β globin fragment, given by K.Nagai, was recloned into Eco RI-linearised pSP65. This plasmid was linearised with Hind III prior to transcription. Both ψO_a and β globin constructs were orientated to give coding sense transcripts.

RESULTS

In order to determine the role of O_a secondary structure elements in the nucleation of TMV assembly, sequences making up hairpin loops in the RNA were deleted from a cDNA clone for O_a, to produce constructs giving

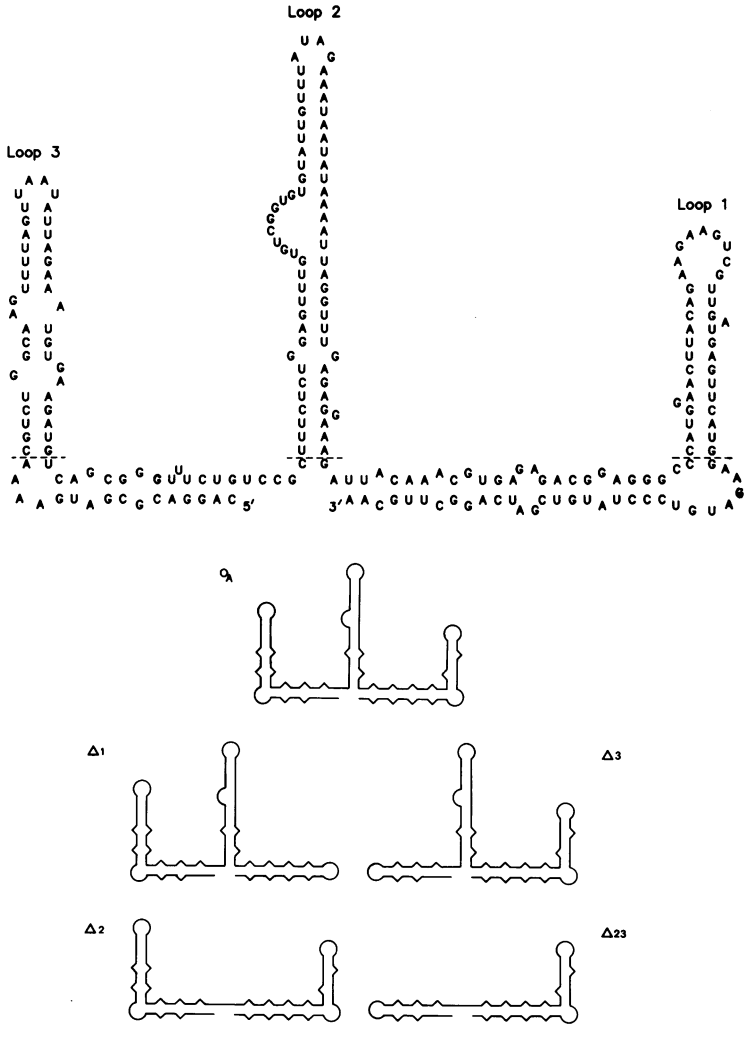

Fig. 1 (a) Proposed secondary structure for the TMV assembly origin (16), extending from bases 5290 to 5527 of the viral RNA sequence. Loop nomenclature is that used in the text; loops 1,2 and 3 are labelled A, B, and C in (16). Dashed lines show the sequence breakpoints in the deletion mutants. (b) Schematic diagram showing the structure and nomenclature of deletion mutants of the TMV assembly origin.

rise to the RNA molecules shown in Figure 1. In vitro transcripts of these clones were then tested for nuclease resistance after packaging by TMV coat protein disks under established assembly conditions. The results are shown in Figure 2. After 10 minutes packaging a full length O_a transcript was

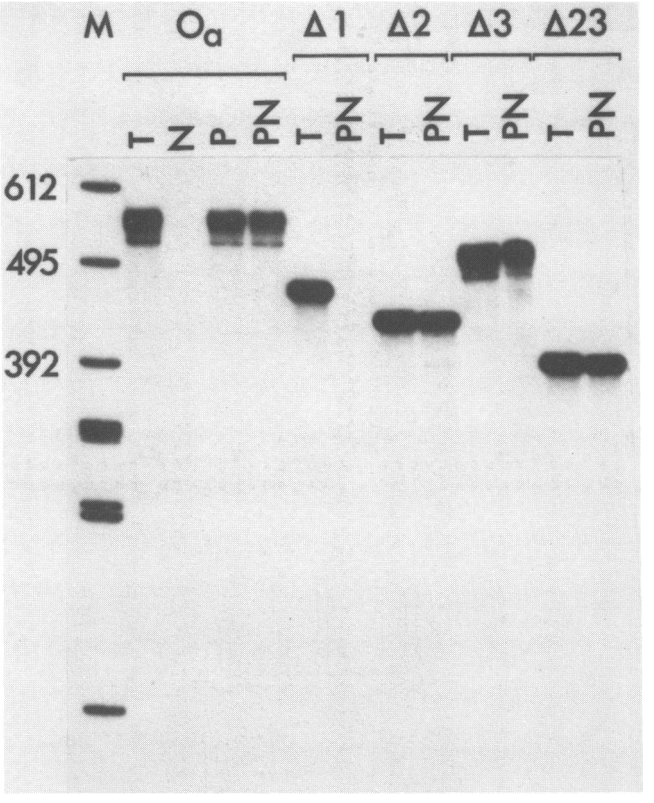

Fig. 2 Disk-dependent protection from nuclease digestion of radioactively labelled assembly origin and deletant transcripts; autoradiogram of 4% denaturing polyacrylamide gel. Lanes T; untreated transcripts. Lane N; nuclease treated transcript. Lane P; disk packaged transcript. Lanes PN; transcripts packaged with disks followed by nuclease treatment. Lane M; approximate size markers, labelled Hinc II-cut ΦX174 DNA. Fragment sizes in bases; 612, 495, 392, 345, 341, 335, 297, 291 and 210.

protected, showing assembly with coat protein to have occurred irrespective of the presence of short polylinker-derived sequences at either end of the RNA. Transcripts of the deletion mutants Δ2, Δ3 and Δ23, lacking loops 2 and 3 singly and together, were also protected. The Δ1 RNA (lacking loop 1, the core of O_a) was not protected; this result therefore serves as an internal control to demonstrate the specificity of the assembly process. The rate of encapsidation was also tested using a nitrocellulose filter binding assay. The O_a transcript was rapidly and quantitatively retained on filters after assembly, as shown in Figure 3. Under the reassembly conditions used, the process is pseudo-first order with respect to RNA

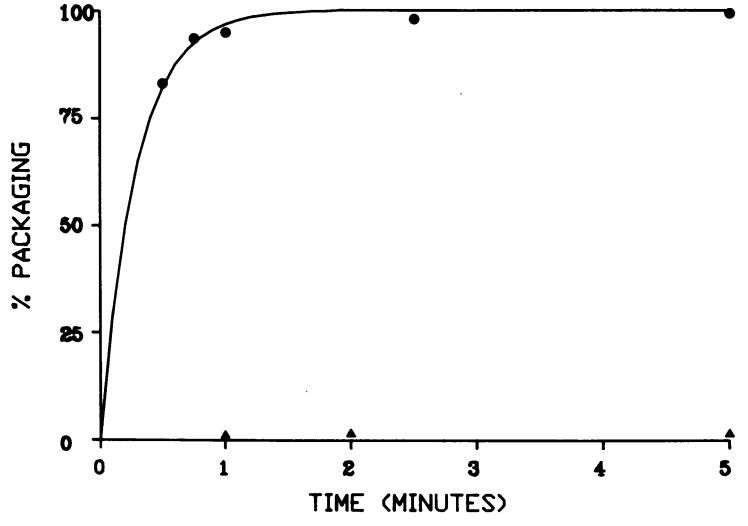

Fig. 3 Packaging rates of assembly origin (●) and Δ1 (▲) transcripts treated with disks as measured by nitrocellulose filter binding. All points are averages of duplicate measurements. The curve fitted to the assembly origin points, for illustrative purposes only, is an exponential with a half time of 12 seconds.

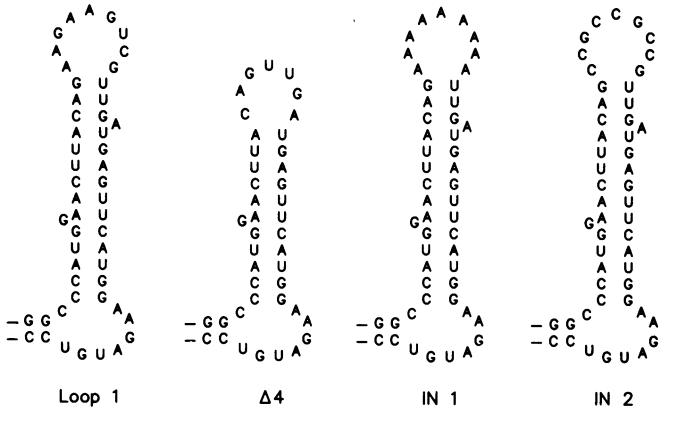

Fig. 4 Structure of assembly origin loop 1 mutant RNA molecules; nomenclature as in the text. The sequences of the mutant transcripts were otherwise unchanged.

concentration with a half time of <30 seconds, as previously shown with labelled fragments of TMV RNA (11). RNA from the Δ2, Δ3 and Δ23 mutants was bound to filters after assembly at a rate indistinguishable from that of O_a (data not shown), whereas Δ1 showed no rapid packaging (but see below).

Loops 2 and 3 can therefore be removed from the RNA fragment without affecting the rate or extent of its packaging by disks. Removal of loop 1 abolishes the packaging reaction, however, correlating with evidence that the assembly process begins at this locus on the intact RNA (12). In order to show that removal of loop 1 does not merely disrupt a distinct disk-binding feature, and to test the hypothesis that the sequence at the tip of loop 1 is instrumental in disk/RNA recognition, cDNA clones producing transcripts with the altered loop 1 structures shown in Figure 4 were constructed. In Δ4 the 9 bases at the apex of loop 1 are deleted, leaving a truncated hairpin. In IN1 a similar sequence lacking the characteristic guanosine residue every three bases is substituted. The G periodicity is retained in IN2, but the other bases have been replaced with C residues; thought to be unfavourable for disk binding due to their rarity in the vicinity of loop 1.

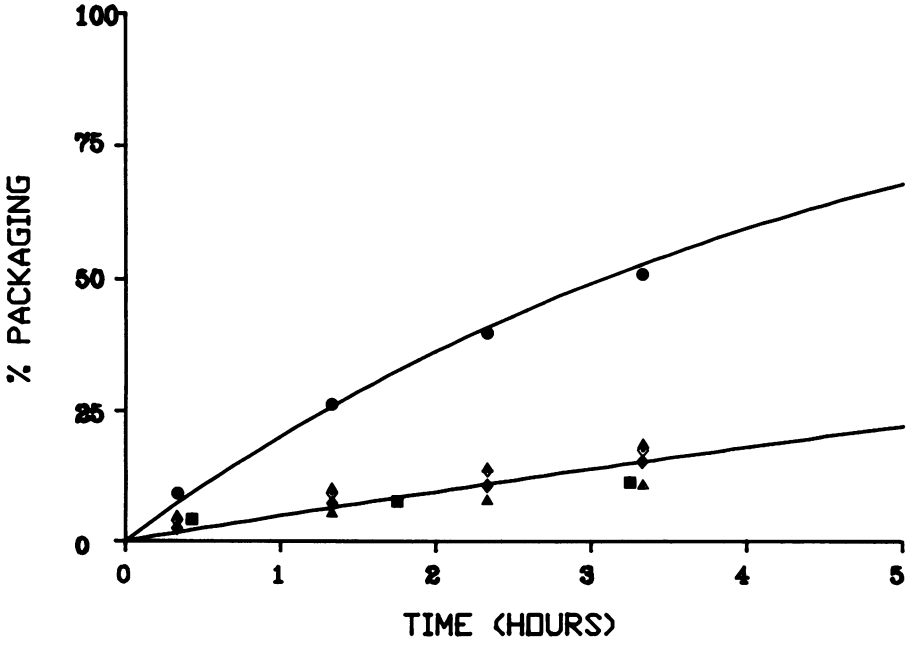

Fig. 5 Packaging rates of assembly origin loop 1 mutant RNAs and other transcripts after treatment with disks; Δ1 (◆), Δ4 (▲), IN1 (◈), IN2 (●), β globin (■) and ψO_a (▲). Data measured by filter binding; all points are averages of duplicate measurements. The curve fitted to the IN2 data is an exponential with a half time of 3 hours; that fitted to the Δ1, Δ4, IN1, β globin and ψO_a data has a half time of 11 hours.

After assembly with disks, transcripts of these constructs were tested for retention on filters. No rapid packaging was found for Δ4, IN1 or IN2, nor were protected RNA fragments detected on gels after nuclease treatment, as found for the Δ1 mutant transcript (Fig. 3). It can therefore be concluded that loop 1, and more precisely the unpaired sequence at its apex, is indeed critical for the nucleation of TMV assembly.

Continuation of the packaging reactions for longer times revealed quantitatively different assembly rates in the filter binding assay. This data is shown in Figure 5 relative to a β globin control transcript. IN2 RNA was packaged most rapidly, with a half time of about 3 hours. Transcripts of the Δ1, Δ4 and IN1 constructs were all filter bound by coat protein disks significantly more slowly, with a half time of around 12 hours. That the latter rate is likely to represent a baseline of nonspecific packaging is shown by the filter binding of the β globin transcript at the same rate.

Are these very slow packaging rates relevant to the authentic TMV assembly process, which is normally complete in a matter of minutes? The intermediate packaging rate of the IN2 transcript is disk-dependent; with the smaller 'A' protein aggregate of TMV protein (24) a much lower, invariant packaging rate was seen with the Δ4, IN1, IN2 and globin transcripts (data not shown). IN2 RNA encapsidation leads to the formation of nuclease resistant protein-RNA complexes which appear as short helical rods in the electron microscope. It is therefore indistinguishable from authentic TMV assembly except by virtue of the considerable difference in rate, and conclusions can be drawn with confidence as to the sequence specificity of disk binding. Packaged Δ1, Δ4 IN1 and IN2 transcripts were found to be nuclease resistant over their full length by gel electrophoresis (data not shown). β Globin and ψO_a (see below) packaged transcripts were not, suggesting that these constructs contain sequence or structural elements which are inhibitory to full length packaging.

As noted above, in the assembly defective TMV mutant Ni 2519, potentially stem-disrupting mutations are found in loop 3, and may account for the observed breakdown in specificity of assembly nucleation at the restrictive temperature (30-35°C) to allow competition from assembly beginning at ψO_a (16,21). In order to rest this idea, a transcript of the TMV coat protein gene including the ψO_a sequence was synthesised and its packaging rate measured by the filter binding assay at room temperature and at 33°C, in parallel with the $O_a\Delta 3$ mutant. At room temperature, ψO_a was

packaged at an apparently non-sequence specific rate (see Figure 5). At 33° the packaging rate increased slightly (data not shown), but its half time remained >6 hours. In contrast, even at the higher temperature Δ3 was still packaged at a rate indistinguishable from that of O_a (i.e. $t_{\frac{1}{2}}$ around 15 seconds). There is therefore no evidence for significant competition with respect to assembly initiation between the $O_a\Delta3$ mutant and ψO_a, at least when present as subgenomic RNA fragments.

DISCUSSION

Role of secondary structure elements in assembly initiation

Previous studies have shown the 3' end of the TMV assembly origin, centred on loop 1, to be the earliest packaged region in virus assembly (12). Other data, however, suggested that loops 2 and 3 might also have a role in assembly nucleation (16). The fact that loops 2 and 3 can be deleted individually or together from a TMV O_a RNA fragment without affecting the rate or extent of its packaging by disks, shows that neither the structures themselves nor sequences within them are required for assembly. Since the overall secondary structure of the transcripts is unlikely to be perturbed by the precise removal of hairpins it seems that the particular loop spacing, which is of course changed in the deletion mutants, is not a key feature in assembly nucleation. These results also rule out a function in disk binding for the UACAAAU sequences present in both loops. A function for sequences outside the deleted elements, or to the 5' of the structure shown in Figure 1, is not formally excluded by this data, but is unlikely in view of the demonstrated origin of assembly at loop 1 (12).

It is possible that by employing a short fragment of TMV RNA in this work, the effect of long range structural interactions on the assembly of the full length genomic RNA has been overlooked. We consider this unlikely owing to the close correspondence between packaging rates of the O_a fragment and whole TMV RNA (11), when compared with control sequences. Indeed, recent evidence shows that the O_a fragment used here can direct the rapid and efficient packaging of contiguous foreign RNA sequences by TMV coat protein disks (22), supporting the original assumption that O_a contains all the signals necessary to direct the initiation of assembly (11).

In the light of these findings, it is now possible to reconsider the observations which suggested the involvement of an extended RNA structure

in assembly nucleation. Assembly of TMV RNA in conditions of limiting protein results in the protection of a (on average) 300 base tract of RNA around O_a. It is not necessary to invoke a concerted recognition process to explain this result, however; it could simply be a consequence of the combined kinetics of assembly initiation & elongation (32). The current results imply that after the initial specific rate-limiting disk/RNA interaction, a nucleoprotein complex containing about 300 bases of RNA is rapidly formed in a sequence and secondary structure-independent fashion. Bidirectional elongation of this complex may then occur to complete assembly. No substitute for the previous model (16) is therefore required.

The TMV mutant Ni 2519 is temperature sensitive both for assembly and for local lesion spreading (33,34). With regard to assembly, at 33°C initiation of packaging at ψO_a appears to compete with that at O_a, leading to the formation of non-infective 'broken rods' of coated RNA (34). Potentially stem weakening mutations in loop 3 were identified (21), but it was unclear whether these mutations are responsible for the defect in assembly, the defect in local lesion spreading (which is thought to be mediated by the TMV 30K protein, whose coding region encompasses O_a) or both (16,21).

When taken together, the observations that loop 3 can be deleted without measurably affecting the rate of packaging of the fragment, even at 33°C, and that a ψO_a fragment shows extremely slow packaging, again even at 33°, lead to the following conclusion. It is possible that the loop 3 mutations in Ni 2519 lead at the restrictive temperature to the formation of an alternative, deleterious secondary structure at O_a. In view of the fact that loop 3 has no active role in assembly initiation, however, it seems more likely that additional mutations exist, possibly at ψO_a, which account for the observed defect in assembly in Ni 2519.

The observation of a very low packaging rate for ψO_a in these experiments was surprising in view of the original definition of ψO_a as specifically encapsidated TMV RNA fragment(s)('SERFs' (19,20)). While the fragment used here was longer than the original ψO_a fragment, it seems likely that the different results are caused by the difference in packaging conditions; 4 hours assembly in ionic strength 0.5M phosphate buffer was used by Guilley and his collaborators. Several other observations of the packaging of foreign RNA molecules have been made using extended incubation periods or high ionic strength buffers (35,36,37) which are likely to overcome the specificity of the assembly initiation process. It is

suggested that an operational definition of authentic assembly nucleation as a disk-dependent filter binding capability with a half time of <30 seconds is more appropriate.

Role of loop 1 in assembly initiation

The abolition of packaging of the Δ1 mutant transcript, then, corroborates the nuclease protection data (12) to establish loop 1 as the primary disk binding site in assembly initiation. A transcript in which the unpaired nonanucleotide sequence at the tip of this hairpin has been removed (Δ4) also fails to show rapid packaging with disks, as do two different transcripts in which five base substitutions have been made in this sequence (IN1 and IN2). These mutations are unlikely to alter the stably base paired structure of the loop 1 stem, and it can therefore be concluded that this sequence is instrumental in disk binding to the TMV O_a.

The results do not exclude a function in assembly for sequence and/or structural features of the stem itself, although any such features are clearly not functional alone. When taken together with evidence that the oligonucleotide AAGAAGUUG binds strongly to disks and causes their aggregation into virus-like helical structures (38), however, these findings suggest that this nine base element contains all the sequence features necessary for specific RNA recognition in the nucleation of TMV assembly. The function of the rest of the hairpin may be to present this sequence to the disk, perhaps in the context of a relatively easily melted hairpin. One noteworthy point is that in the Δ1 RNA a stem with the sequence CCGAAGAUGU at the apex is created across the deleted region (see the base of loop 1 in Figure 1). Despite the similarity of this sequence to the natural loop 1 apex, no rapid disk binding is seen. This supports speculation that the length of loop 1, and/or the marginally stable base pairing close to its apex are important structural features in the RNA-protein interaction.

Observation of differential packaging rates for the Δ4, IN1 and IN2 transcripts allows certain conclusions to be drawn as to the relative contributions of aspects of the loop 1 sequence feature to the rate of assembly. The $(A)_9$ sequence substituted at the loop 1 apex in the IN1 RNA evokes no more rapid packaging than transcripts lacking this sequence altogether, or to control transcripts. Since the ability of TMV disks to encapsidate poly(A) is established (6,39,40), this result supports the idea that poly(A) packaging occurs by a different mechanism from that by which TMV RNA is coated (32,41). The IN2 transcript is packaged significantly

more rapidly than IN1 RNA, but much less rapidly the wild type O_a fragment. This suggests that the 3 base G periodicity at the apex of loop 1 is an important feature in disk binding, as anticipated (12,14), but further work will be required to confirm this point.

The sequence specificity of trinucleotide binding to TMV protein in helical form has been investigated previously (42). It is not clear that these earlier results are relevant to the assembly initiation process (when the protein is required to be in a radically different conformational state (6)). It is, however, worthwhile to note that AAA showed no binding and CAG/ACG showed reduced binding in comparison with the most strongly binding trimer AAG, in apparent agreement with the current findings.

Experiments are in progress using the methods outlined in this paper to further characterise the sequence and structural basis of RNA recognition by disks in the TMV assembly nucleation process.

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